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Survival of *Arthrobacter crystallopoietes* During Prolonged Periods of Extreme Desiccation

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Cells of *Arthrobacter crystallopoietes* mixed in sand and air-dried have survived for up to 6 months after an initial period in which approximately half the cells lost their viability. Comparative survival curves have been obtained from inoculated sands maintained under $CaSO_4$ or P_2O_5. Selections for more desiccation-resistant progeny capable of surviving the initial period were unsuccessful. Both the coccoid and rod-shaped forms are equally resistant to several months of desiccation. Desiccated spherical cells converted 0.0005% of their cell carbon to carbon dioxide per hr, which corresponds to a half-life for self-consumption of approximately 12 years.

Estimations of the number of bacteria in soil range from 10^6 to 10^9 per g. These organisms perpetuate themselves in a habitat which is repeatedly subjected to cycles of drying and wetting. The impact of these natural processes on the soil microflora is not clearly understood. Clark stated that all soil bacteria are resistant to drying, and individual species are seldom eliminated from air-dried soils (6). However, as a soil persists in a dry state, there is a progressive decrease in the numbers and kinds of microorganisms which are able to survive, in favor of those capable of forming resistant cysts and spores. *Azotobacter* cysts have survived 30 years in soil air-dried in the laboratory (6). *Streptomyces* spp. were isolated from soil attached to herbarium samples that date from 1850, and *Bacillus* spp. were isolated from soil attached to herbarium specimens collected before 1700 (15).

There is very little in the recent literature pertaining to the drought resistance of common soil organisms which are not known to form specialized resting stages. Unidentified species of *Arthrobacter* have been isolated after several months from soils dried to ambient humidity (11, 14) or soils dried under $CaSO_4$ (M. Chen and M. Alexander, Bacteriol. Proc., p. 1, 1970). *Arthrobacter* spp. and other globiforme bacteria found in Antarctic desert soils can be cultured after the soils have undergone 3 years of laboratory drying (5).

Members of the genus *Arthrobacter* appear to be the most numerous single group of bacteria in soils (6, 11, 12), and, depending upon the locality, they represent from 5 to 35% of the isolatable colonies (1). Desiccation resistance of *Arthrobacter* may partly explain their numerical dominance in soil (14). A second reason postulated for their relative abundance is that they are inherently resistant to nutrient depletion, a condition encountered in soils (4). Cells of one species, *A. crystallopoietes*, have been shown to withstand starvation in phosphate buffer for 30 days without loss in viability (3, 4).

Bacteria of the genus *Arthrobacter* are characterized by their unique ability to grow as two distinct cell shapes, forming either spherical or rod-shaped cells, depending upon culture medium and growth phase (4, 10). It is believed that the spherical form predominates in soil (7, 11), and, therefore, it has become generally accepted as the more resistant of the two morphological forms (11).

This study was made to assess the ability of *A. crystallopoietes* to survive extended periods of drought and to determine the relative resistance to desiccation of the spherical and rod-shaped forms of this organism.

MATERIALS AND METHODS

Growth conditions. *A. crystallopoietes* (ATCC-15481) was grown as spheres in the glucose-mineral salts (GS) medium previously described (4). Rod-stage cells were grown in a medium of 1% peptone and 0.1% yeast extract. *Pseudomonas diminuta* (ATCC-11568) and *Bacillus subtilis* (ATCC-6051) were also grown in this medium. All cells were grown at 30 C on a gyratory shaker and

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harvested during the exponential phase. Sporulated cultures of *B. subtilis* were obtained after 40 hr.

Desiccation conditions. All cells were washed in 0.03 M potassium phosphate buffer, pH 7.0, and suspended in the same buffer. The cells were diluted in the same buffer such that soil inoculations were approximately 10^6 cells per g of soil. Cells were distributed into tubes containing 1 g of sterile sand with low organic content (Death Valley desert sand containing <0.2 mg of organic carbon per g of sand) or 1 g of sterile, acid-cleaned 100-μm glass beads (13). Sand or glass beads were used to adsorb the liquid from the inoculations and to distribute the cells as freely as possible.

The release of cells in buffer which had been freshly distributed through either glass beads or sand grains was facilitated by a mechanical mixer (Vortex). Viable cell counts were comparable for periods of mixing from 30 sec to 10 min, and were in agreement with plate counts made on the same cell suspensions prior to inoculation into the solid milieu. Resuspension in buffer of cells which had been dried for 2 days in either sand or glass beads gave similar colony counts when mixing was done for periods of 30 sec to 10 min. Static resuspension (no agitation) or agitation for only 15 sec gave repeatedly lower plate counts with freshly distributed cells or cells dried for 2 days. Mixing for 30 sec was subsequently used throughout the study. Any decrease in plate counts with length of drying, therefore, was attributed to an actual decrease in viability and not to an increase in adherence of the cells to the solid particles.

The inoculated tubes were dried to ambient humidity (25–50% relative humidity) at 35 C for 12 hr prior to storage at room temperature. At intervals, the cells in three tubes were suspended and diluted in buffer, and triplicate platings on nutrient agar (fortified with 0.3% yeast extract) were made from each tube. To achieve lower humidity conditions, inoculated tubes were incubated over the desiccants, $CaSO_4$ or P_2O_5, at one atmospheric pressure.

Respiration studies. The endogenous respiration rate of cells subjected to desiccation conditions was measured by determining the amount of $^{14}CO_2$ released from labeled cells. Spherical cells were grown in the GS medium, in the presence of glucose-*UL-*^{14}C (specific activity 500 mCi/mmole; 1.0 μCi/ml culture) for 36 hr (3 cell generations). Cells (100-ml suspension) were washed twice and suspended in 2.5 ml of buffer. The cells in 0.5 ml of this suspension were distributed throughout 30 g of sterile glass beads which were then layered onto a sintered glass filter contained in the drying chamber (Fig. 1). Duplicate chambers served as the experiment, and a chamber containing the same number of labeled cells previously heated in a boiling water bath for 15 min served as the control. Each chamber contained approximately 3×10^{10} cells which possessed a total radioactivity of 6.6×10^6 disintegrations per min. Chambers were maintained at ambient temperature and atmosphere. Air, dried by serial passage through three concentrated H_2SO_4 bubbling towers, was continuously passed through the glass beads and bubbled through a train of three vials each holding 10 ml of

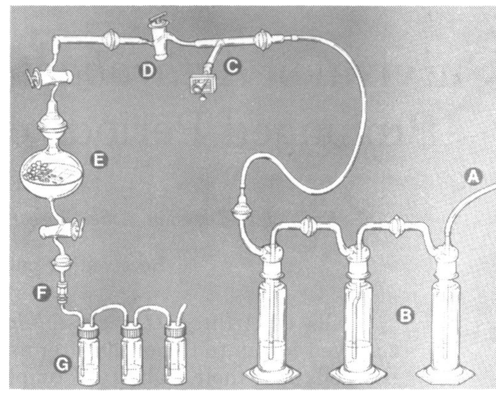

FIG. 1. *Apparatus to measure the respiration rate of Arthrobacter crystallopoietes in the dry state. Symbols: A, air hose leading from air supply; B, conc. H_2SO_4 bubbling towers to dry the air; C, pressure release valve; D, vacuum stopcock; E, incubation chamber with sintered glass partition; F, reduction gas fitting; G, scintillation vial train for trapping CO_2.*

scintillation fluid containing phenethylamine (16). Sampling vials were removed at intervals for counting and replaced by vials with fresh scintillation fluid. The trapped CO_2 was then counted for radioactivity in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The radioactive glucose was purchased from New England Nuclear Corp. and used without further purification.

RESULTS

When harvested during exponential growth and dried to ambient laboratory humidity, sperical cells of *A. crystallopoietes* were capable of surviving desiccated conditions for periods up to 6 months after an initial period in which approximately one-half of the cells lost their viability (Fig. 2). Although no exact measures of decreasing humidity during the initial flushing period were taken, the sand was observed to become dry; the 2-day period in which the wetness disappeared corresponded with the initial loss of viability. Viability determinations were not continued beyond 6 months.

The survival curve for spores of *B. subtilis* remained unchanged for the duration of the experiment (Fig. 2). By comparison, when vegetative cells of *B. subtilis* or *P. diminuta* (both common soil organisms) were dried in either glass beads or sand, loss of viability was rapid. In less than 12 hr, a viable population of 10^6 cells was reduced to a population of several hundred. Since the loss in viability was 4 logs, viability was not further assessed after 24 hr.

To verify that this observed decrease in

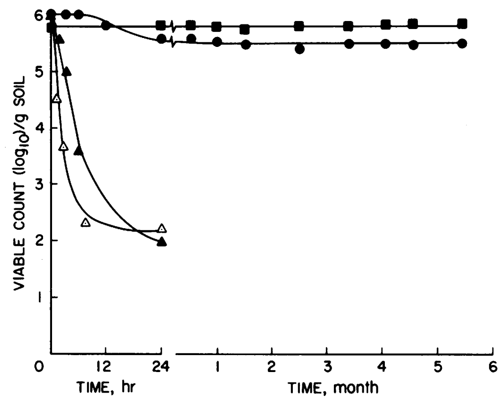

FIG. 2. *Viability of different organisms dried to ambient humidity. Cells were suspended in buffer and inoculated into tubes of sterile desert sand. Soil tubes were dried to ambient laboratory humidity and temperature. Symbols:* ■, *Bacillus subtilis spores;* ●, *Arthrobacter crystallopoietes vegetative cells;* ▲, *B. subtilis vegetative cells;* △, *Pseudomonas diminuta vegetative cells.*

viability was real and not a preferential adherence to the glass beads or sand grains by cells of the various species, separately washed cell suspensions were distributed on 0.45-μm filters. The filters were allowed to dry gradually under ambient conditions in glass dishes. At various times, the filters were remoistened by being placed on the surface of nutrient agar and incubated at 30 C until colonies were large enough to count. Although this procedure was abandoned because the low number of viable cells per filter necessitated using many filters for proper statistical evaluations, the trend for each death curve shown in Fig. 2 held true for each organism.

Survival of cells of *A. crystallopoietes* dried over $CaSO_4$ or P_2O_5 is shown in Table 1. For periods of up to 28 days, cells of *A. crystallopoietes* showed similar resistance to varying degrees of dryness. Cell viability was the same whether the cells were dried in sand or glass beads.

The possibility that the initial drop in viability shown by cells of *A. crystallopoietes* was due to selection between desiccation-susceptible and desiccation-resistant organisms was investigated. Cells surviving 24 hr of drying were reinoculated into GS medium and incubated at 30 C for 3 days. Samples of these progeny were dried in soil tubes for 24 hr. Cells surviving after 24 hr were again inoculated in GS medium. This procedure was repeated for five cycles. In each instance, the viable cell count after 24 hr was approximately one-half that of zero time.

TABLE 1. *Viability of Arthrobacter crystallopoietes under varying degrees of desiccation*

Duration of desiccation (days)	Cells dried to ambient humidity		Cells dried over $CaSO_4$	Cells dried over P_2O_5
	Sand	Glass beads	Glass beads	Glass beads
0	2.2[a]	2.1	2.1	2.1
1	1.5	1.6	1.1	0.9
28	1.1	0.7	0.8	0.5

[a] Colony-forming count per gram of sand or glass beads $\times 10^6$.

No selection of completely resistant progeny could be obtained.

Rod-stage cells of *A. crystallopoietes* were subjected to desiccation conditions similar to those imposed on the spherically shaped cells. As with the spheres, a similar initial drop in viability during the first 24 hr of drying was observed. Thereafter, no significant decrease in viability occurred during a 6-month drying period. Survival curves were similar to those obtained with spherical cell cultures.

The results of the respiration experiment are shown in Fig. 3. The release of $^{14}CO_2$ from spheres was rapid during the first few hours of starvation. From 0.2 to 0.6% of cell carbon was released as CO_2 each hr during the first day of drying. Thereafter, the rate at which the cells respired $^{14}CO_2$ decreased rapidly. The rate continued to decrease until a constant rate was achieved after 4 days of desiccation. The basal level of endogenous metabolism corresponded to the utilization of 0.0005% of the total cell carbon per hr. The heat-killed cells which served as a control for this experiment initially gave off 0.001% CO_2, presumably as a product of chemical decomposition or autoradiolysis. Nonbiological release of CO_2 from heat-killed cells was noted by other investigators (9). This rate dropped rapidly until after 4 days a constant rate of 0.00002% CO_2 was released per hr. Control values constituted approximately 4% of the rate obtained for viable cells at any single time during the experiment. Experimentally, these rates were 20 to 30 disintegrations per min per hr for the viable cells and 1 to 2 disintegrations per min released per hr for the sterile control. Sampling was done at approximately 12-hr intervals to ensure a statistically significant release of $^{14}CO_2$.

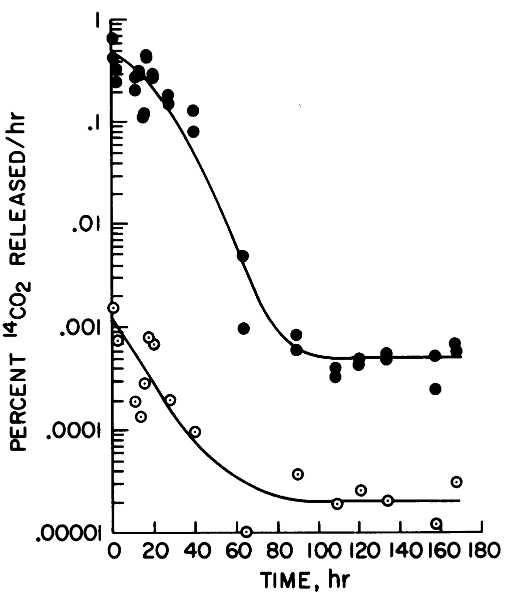

FIG. 3. *Rate of $^{14}CO_2$ evolution from desiccated spherical stage cells of Arthrobacter crystallopoietes grown on radioactive glucose. Cells grown in GS medium containing glucose-UL-^{14}C for 36 hr were harvested and resuspended in buffer. The cells were dispersed into glass beads and put in special drying chambers. The $^{14}CO_2$ was collected and counted. Symbols: ●, percentage of original label in spherical cells released as $^{14}CO_2$; O, percentage of original label in heat-killed cells released as $^{14}CO_2$.*

DISCUSSION

The parameters set for the experiments discussed in this paper were chosen to simulate naturally occuring drought conditions in an effort to assess the resistance of certain soil microorganisms when exposed to extremely dry environments. Arthrobacters, by their sheer numbers and varied physiological activities, may exert a strong influence on microbiological processes in the soil. As suggested by Robinson et al. (14), the advantage enjoyed by arthrobacters in soil may reflect, in part, their ability to withstand drying, a common process to which soils are subjected. The data presented in this report support that conclusion.

Although members of the genus *Arthrobacter* show a morphological variation from coccoid to rod-like, only the coccoid form of arthrobacters have been observed in soils (6, 7, 11). Consequently, the persistence of these organisms in soils in the coccoid form was presumed to be due to a special property of this form (11). In the past, the difficulty in preparing rod and sphere cultures in the growth phase of older known species of *Arthrobacter* made direct experimental analysis of this assumption difficult. Because morphogenesis in *A. crystallopoietes* can be nutritionally controlled (10), the relative resistance of the coccoid and rod forms could be tested experimentally. Both morphological forms of *Arthrobacter* were equally resistant to desiccation under the conditions of our experiments. If arthrobacters can grow as rods in nature, they must exist as rods only transitionally, returning to the spherical form either before nutrients are totally depleted or before desiccation of the soil becomes sufficient to halt growth. It is more likely, however, that the nutrients necessary for the expression of the rods may never be available in sufficient concentrations in soils. Even though rod forms of *Arthrobacter*, when in the process of reductive division to coccoid cells, are not as resistant to desiccation (Boylen, *unpublished data*), drying of subsurface soil layers may take as long as several days, although surface layers can dry out in several hours in sunlight, while most *Arthrobacter* species can complete a sphere to rod to sphere growth cycle within a few hours. Populations of stationary growth phase spheres exposed to drying never survived as well as populations of growing spheres which underwent desiccation. Recovery of viable cells after 2 days of drying was less than 10% of the initial number inoculated into glass beads (Boylen, *unpublished data*).

We noted that the recovery of viable organisms immediately after drying was approximately one-half of the initial number. Similar observations were made by Clark (6) as to the reduction in the number of bacteria occurring naturally in soil as it approaches an ambient dry state, and by Annear and Bottomley (2), who reported about 50% recovery of viable dried organisms immediately after the initial drying was completed. In *A. crystallopoietes*, the initial loss in total viable count is not merely a response by desiccation-susceptible cells, since attempts to select for a completely resistant clone were unsuccessful. However, Chen and Alexander (Bacteriol. Proc., p. 1, 1970) reported that *Arthrobacter* spp. soil isolates can be selected to yield more desiccation-resistant progeny.

The basal level of endogenous metabolism of spherical *Arthrobacter* cells under the specific desiccating condition investigated represents the conversion of 0.0005% of cell carbon to carbon dioxide per hr. This corresponds to a half-life for self-consumption of approximately 12 years. No evidence is presented to directly relate the longevity of dried cells to their endogenous metabolism. However, Cameron and Conrow (5) recovered *Arthrobacter* spp. from Antarctic desert soils after 4 years of laboratory desiccation. The metabolic rate determined in our study is the lowest yet reported for vegetative bacterial cells. By comparison, the basal level for cells of *A. crystallopoietes* during starvation in aqueous buffer was reported to be 0.03% of the cell carbon per hr, to give a half-life for carbon utilization of 70 days (4). By further comparison the determinations of Desser and Broda (8, 9) for the endogenous metabolism rates of *Bacillus* spores correpond to a half-life for self-consumption of hundreds of years.

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